Data Summary and Review on the Toxicity of AE C656948 (Fluopyram) to Fathead Minnow (*Pimephales promelas*), Early Life Cycle

PMRA Submission Number {.....} EPA MRID Number 473723-36

Data Requirement:	PMRA Data Code	{.....}
	EPA DP Barcode	353315
	OECD Data Point	{.....}
	EPA MRID	473723-36
	EPA Guideline	850.1400

Test material: AE C656948 **Purity:** 94.7%

Common name Fluopyram

Chemical name:

IUPAC: N-{2-[3-chloro-5-(trifluoromethyl)-2-pyridyl]ethyl}-α,α,α-trifluoro-o-toluamide

CAS: N-[2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]ethyl]-2-(trifluoromethyl)benzamide

CAS No.: 658066-35-4

Synonyms: None reported

Reference/Submission No.: {.....}

Company Code	{.....}	[For PMRA]
Active Code	{.....}	[For PMRA]
Use Site Category	{.....}	[For PMRA]
EPA PC Code	080302	

CITATION: Nieden, D. 2006. Early-life Stage Toxicity of AE C656948 (tech.) to Fish (*Pimephales promelas*). Unpublished study performed and sponsored by Bayer CropScience AG, D-40789 Monheim, Germany. Laboratory Study No. E 284 3156-9. Study initiated May 15, 2006 and submitted October 23, 2006.

EXECUTIVE SUMMARY:

The 33-day chronic toxicity of AE C656948 (fluopyram) to the early life stage of fathead minnow (*Pimephales promelas*) was studied under flow-through conditions. Fertilized eggs/embryos (100/level, <24 hours old) of fathead minnow were exposed to AE C656948 at nominal concentrations of 0 (negative and 0.1 mL/L DMF solvent controls), 0.0185, 0.0370, 0.0740, 0.148, 0.296, 0.592, and 1.18 mg ai/L (adjusted for purity). Reviewer-calculated TWA concentrations were <0.00514 (<LOQ, controls), 0.0175, 0.0380, 0.0652, 0.135, 0.269, 0.560, and 1.05 mg ai/L, respectively. The test system was maintained at 24.7 to 26.8°C and a pH of 6.5 to 7.4. The 33-day NOAEC and LOAEC values were 0.135 and 0.269 mg ai/L, respectively, based on clinical signs of toxicity, the most sensitive endpoint.

The time to hatch and hatching success were unaffected by exposure. For all levels, hatching occurred from days 3 to 5, and hatching success ranged from 89 to 95%. Prior to thinning (on day 5), larval mortality (5%) was observed at the 1.05 mg ai/L treatment level and was considered a result of exposure; no other larval mortality occurred at any level. Treatment-related signs of toxicity were observed at the ≥0.269 mg ai/L levels from days 20 to 33; effects included deformed mouth, ventral hematoma, labored respiration, remaining at the water surface, dark coloration, swollen belly, loss of equilibrium (with lateral deviation from normal orientation), and/or lordosis.

A statistically-significant reduction in post-hatch survival was indicated at the two highest treatment levels compared to the negative control; mean fry survival (day 33) ranged from 87 to 97% for the control through 0.269 mg ai/L levels, 70% at the 0.560 mg ai/L level, and 15% at the 1.05 mg ai/L level. The growth of surviving fry was adversely affected by exposure. Mean lengths were statistically-reduced compared to the negative control (21.0 mm) at the 0.560 and 1.05 mg ai/L levels (16.7 and 10.8 mm, respectively). Mean dry weights were also statistically-reduced compared to the negative control (35.1 mg) at the 0.560 and 1.05 mg ai/L levels (22.1 and 4.1 mg, respectively).

This toxicity study is scientifically sound/unsound and {does or does not} satisfy the guideline requirement for an early life toxicity study with fish.

Results Synopsis

Test Organism Size/Age(mean Weight or Length): Embryos, <24 hours old
Test Type (Flow-through, Static, Static Renewal): Flow-through

LOAEC: 0.269 mg ai/L
NOAEC: 0.135 mg ai/L

Endpoint(s) Affected: larval survival (day 5), fry survival (day 33), morphological and/or behavioral effects, and growth (length and dry weight)
Most sensitive endpoint(s): morphological and/or behavioral effects

I. REPORTED MATERIALS AND METHODS

GUIDELINE FOLLOWED: U.S. EPA SEP 560/6-82-002 (1982)
ASTM E 1241-92 (1992)
U.S. EPA OPPTS 850.1400 (1996)
OECD Guideline No. 210 (1992)

COMPLIANCE: Signed and dated GLP, Quality Assurance, and Data Confidentiality claims statements were provided. This study was conducted in compliance with the current OECD Principles of GLP and with the Principles of GLP according to Annex 1 of the German Chemical Law (ChemG; 2002) with the following exception: deionized water quality measurements for residues and contaminants.

A. REPORTED MATERIALS:

1. Test Material

Table 1: Test material.

Parameter	Details
Name	AE C656948 (fluopyram)
Description	Beige powder
Lot No./Batch No.	MIX-Batch: 08528/0002
Purity	94.7%
Stability under test conditions	Stable, as indicated by relatively constant (within 20% of mean) measured concentrations determined on days 0, 7, 14, 21, 28, and 33.
Storage conditions	Ambient

Table 2: Physicochemical properties of fluopyram.

Parameter	Values
Water solubility at 20°C	16 mg/L (MRID 473723-40)
Vapor pressure	Not reported
UV absorption	Not reported
pKa	Not reported
Kow	Not reported

2. Test organism:

Table 3: Test organism.

Parameter	Details
Species:	Fathead minnow (*Pimephales promelas*)
Age:	Embryos, <24-hours old
Method of collection of the fertilized eggs:	Freshly-fertilized eggs from seven breeding tanks (one male and two female per tank); not otherwise specified
Source:	Laboratory breeding stock (batch F 5/05)

B. REPORTED STUDY DESIGN:

1. Experimental Conditions

a. Range-finding study: None reported.

b. Definitive study

Table 4: Experimental Parameters

Parameter	Details
Parental acclimation, if any Period: Conditions (same as test or not): Feeding (type, source, amount given, frequency): Health: (any mortality observed)	 Continuously-maintained brood stock Same as test Not reported Not reported
Number of fertilized eggs/embryos in each treatment at test initiation	100 embryos/treatment level: 25 embryos/cup, 1 cup/aquarium, and 4 replicate aquaria/treatment
Concentration of test material nominal: mean-measured: TWA (reviewer-calculated):	 0 (negative and solvent controls), 0.0185, 0.0370, 0.0740, 0.148, 0.296, 0.592, and 1.18 mg ai/L (adjusted for purity) <0.00514 (<LOQ, controls), 0.0176, 0.0380, 0.0647, 0.135, 0.271, 0.570, and 1.05 mg ai/L, respectively <0.00514 (<LOQ, controls), 0.0175, 0.0380, 0.0652, 0.135, 0.269, 0.560, and 1.05 mg ai/L, respectively
Solvent (type, percentage, if used)	Dimethylformamide, 0.1 ml/L
Number of replicates control: solvent control: treated ones:	 4 4 4/level
Test condition static renewal/flow-through: type of dilution system for flow through method: flow rate: renewal rate for static renewal:	 Flow-through Periodic-introduction, continuous flow diluter 12 volume additions/day N/A
Aeration, if any	None reported

Parameter	Details
Duration of the test	33 days (28-days post-hatch)
Embryo cups, if used type/material (glass/stainless steel): size: fill volume:	Embryo cups were oscillated vertically (2 times per minute) Stainless steel pipes with a screened stainless steel bottom perforated with holes (0.8-mm hole diameter) 5.5-cm diameter (length not reported) Not reported
Test vessel type/material: (glass/stainless steel) size: fill volume:	 Glass 12.5 cm x 14 cm with a water depth of 21.5 cm 3.75 L
Source of dilution water	Reconstituted water was prepared by adding salt stock solutions to de-mineralized water (conductivity <0.2 µS/cm). The water was aerated to oxygen saturation prior to use.

Parameter	Details
Water parameters	
hardness:	2.4 to 2.6 °dH (42.72 to 46.28 mg/L as $CaCO_3$)
pH:	6.5 to 7.4
dissolved oxygen:	68 to 105% saturation
temperature (s) (record all the temperatures used for different life stages):	24.7 to 26.8°C (not altered for different life stages)
salinity (for marine or estuarine species):	N/A
other measurements:	Conductivity: 93 to 163 µS/cm
interval of water quality measurements:	Temperature, DO, and pH were measured from alternating vessels (all levels) on days 0, 7, 14, 21, 28, and 33. Temperature was also measured hourly in one control aquarium. Hardness was measured at the negative control, 0.0176, 0.0647, and 1.05 mg ai/L levels (based on mean-measured concentrations) at 0 and 33 days.
Photoperiod	16 hours light/8 hours dark, with 30-minute transition periods Light intensity – 253 to 304 lux
Post-hatch details when the post-hatch period began:	Day 5 (post-hatch day 0)
number of hatched eggs (alevins)/ treatment released to the test chamber:	Following completion of hatch (day 5), fish were impartially thinned to 15 larvae per replicate (60/level)
on what day, the alevins were released from the incubation cups to the test chamber:	Day of hatch

Parameter	Details
Post-hatch Feeding start date:	Shortly after hatch
type/source of feed:	Newly-hatched (<24 hours old) live brine shrimp nauplii
amount given:	*ad libitum*
frequency of feeding:	Two to four times daily until 1 day prior to study termination. All aquaria were siphoned in order to remove excess fecal material and uneaten food.
Stability of chemical in the test system	Stable, as indicated by relatively constant (within 20% of mean) measured concentrations determined on days 0, 7, 14, 21, 28, and 33.
Recovery of chemical:	87 to 103% of nominal (based on mean-measured concentrations)
Frequency of measurement:	Days 0, 7, 14, 21, 28, and 33
LOD: LOQ:	Not reported 0.00514 mg ai/L
Positive control {if used, indicate the chemical and concentrations}	N/A
Fertilization success study, if any number of eggs used: on what day the eggs were removed to check the embryonic development:	N/A
Other parameters, if any	N/A

2. Observations:

Table 5: Observations

Parameters	Details
Parameters measured including the sub-lethal effects/toxicity symptoms	- Time to hatch - Hatching success - Fry survival - Overall survival - Measurement of growth (standard length and dry weight) - Morphological and behavioral effects
Observation intervals/dates for: egg mortality: no. of eggs hatched: mortality of fry (e.g.,alevins): swim-up behavior: growth measurements: embryonic development: other sublethal effects	 Daily Daily Daily N/A Day 33 Not determined Daily
Water quality was acceptable (Yes/No)	Yes
Were raw data included?	Yes
Other observations, if any	N/A

II. REPORTED RESULTS

A. REPORTED MORTALITY:

Hatching success was unaffected by exposure; by day 5, hatching success averaged 89 to 95% for all control and treatment levels. On Day 33 (28 days post-hatch), post-hatch survival averaged 97% for the negative control group, 93% for the solvent control group, and 92, 88, 93, 90, 87, 70, and 15% for the mean-measured 0.0176, 0.0380, 0.0647, 0.135, 0.271, 0.570, and 1.05 mg ai/L groups, respectively. The study author's analysis detected that the differences were statistically-significant from the pooled control (α=0.05) at the 0.570 and 1.05 mg ai/L levels. The study author reported that the NOAEC and LOAEC for survival, based on reductions in post-hatch survival, were 0.271 and 0.570 mg ai/L, respectively. A LC_{50} was not reported.

Table 6: Effect of AE C656948 (fluopyram) on egg hatching and survival at different life stage of fish.

Treatment (mg ai/L) Measured (and nominal) concentrations	Egg hatched/embryo viability on day 5			Time to hatch, % hatched			Juvenile-survival on day 33	
	No. of eggs at study initiation	hatch/embryo viability		Day 3	Day 4	Day 5	No. alive	% survival
		No.	%					
Control (dilution water only)	100	95	95	4	74	95	58	97
Solvent control	100	87	87	0	81	87	56	93
0.0176 (0.0185)	100	89	89	0	77	89	55	92
0.0380 (0.0370)	100	90	90	1	74	90	53	88
0.0647 (0.0740)	100	91	91	1	87	91	56	93
0.135 (0.148)	100	93	93	2	84	93	54	90
0.271 (0.296)	100	92	92	0	91*	92	52	87
0.570 (0.592)	100	95	95	5	95*	95	42	70*
1.05 (1.18)	100	92	92	2	92*	92[(a)]	9	15*
NOAEC		1.05 mg ai/L		1.05 mg ai/L			0.271 mg ai/L	
LOAEC		>1.05 mg ai/L		>1.05 mg ai/L			0.570 mg ai/L	
EC_{50}		N/R		N/R			N/R	
Positive control, if used	N/A							

N/A – Not applicable; N/R – Not reported
[(a)] Five of the hatched larvae were found dead on day 5, resulting in a statistically-significant increase in larval mortality on day 5 compared to the pooled control ($\alpha = 0.05$). Hatched larval survival was 100% at all other control and treatment levels on day 5 (prior to thinning).
* Statistically-significant difference from pooled control ($\alpha = 0.05$); determined by the study author's analysis.

B. REPORTED SUB-LETHAL TOXICITY AND OTHER CHRONIC EFFECTS:

Egg hatching occurred from days 3 to 5, with no statistically-significant differences observed (on percent hatch per day) at the beginning or end of hatch. The study author reported a statistically-significant increase in percent hatch was indicated on day 4 at the three highest treatment levels compared to the pooled control (91-95% compared to 78% for the pooled control; $\alpha = 0.05$). These differences, however, were interim and transient and not considered to be biologically significant, and the NOAEC for time to hatch was 1.05 mg ai/L, the highest concentration tested.

At study termination (day 33), length averaged 20.9, 21.0, 21.4, 21.4, 21.1, 20.9, 19.9, 16.7, and 10.8 mm for the negative control, solvent control, 0.0167, 0.0380, 0.0647, 0.135, 0.271, 0.570, and 1.05 mg ai/L treatment levels, respectively (mean-measured concentrations).

The study author determined that differences in length were statistically-significant ($\alpha = 0.05$) at the three highest treatment levels compared to the pooled control (21.0 mm). Dry weight averaged 35.1, 36.8, 39.5, 41.5, 38.1, 39.7, 36.5, 22.1, and 4.1 mg, respectively, with statistically-significant differences ($\alpha = 0.05$) from the pooled control (36.0 mg) at the two highest treatment levels. The study author reported that based on length, the NOAEC and LOAEC levels were 0.135 and 0.271 mg ai/L, respectively. EC_{50} values were not reported.

Table 7: Effect of AE C656948 (fluopyram) on growth of juvenile fish.

Treatment (mg ai/L) Measured (and nominal) concentrations	Swim-up[(a)]			Growth - length (mm)	Growth-wet weight (mg)	Growth-dry weight (mg)
	day x1	day x2	day xn			
Control (dilution water only)	N/A	N/A	N/A	20.9	152.9	35.1
Solvent control	N/A	N/A	N/A	21.0	162.3	36.8
0.0176 (0.0185)	N/A	N/A	N/A	21.4	N/D	39.5
0.0380 (0.0370)	N/A	N/A	N/A	21.4	N/D	41.5
0.0647 (0.0740)	N/A	N/A	N/A	21.1	N/D	38.1
0.135 (0.148)	N/A	N/A	N/A	20.9	N/D	39.7
0.271 (0.296)	N/A	N/A	N/A	19.9*	N/D	36.5
0.570 (0.592)	N/A	N/A	N/A	16.7*	N/D	22.1*
1.05 (1.18)	N/A	N/A	N/A	10.8*	N/D	4.1*
NOAEC	N/A			0.135 mg ai/L	N/A	0.271 mg ai/L
LOAEC	N/A			0.271 mg ai/L	N/A	0.570 mg ai/L
EC_{50}	N/A			N/R	N/A	N/R

[(a)] Swim-up is not applicable for this species.
N/A – Not applicable; N/R – Not reported; N/D – Not determined
* Statistically-significant difference from pooled control using Williams Test (α=0.05); determined by the study author.

During the post-hatch period between days 9 and 18, abnormalities included a swollen belly, lordosis, kyphosis, scoliosis, pale coloration, loss of equilibration (with lateral deviation from normal orientation), laying inactive on the bottom of the vessel, laying on sides or back, abnormal vertical positioning, and/or remaining at the water surface. These observations occurred sporadically in controls and nearly all test concentrations and were not considered by the study author to be related to treatment. No clinical effects were observed at any level on day 19. During the post-hatch period between days 20 and 33 (test termination), abnormalities included deformed mouth, ventral hematoma, labored respiration, remaining at the water surface, dark coloration, swollen belly, loss of equilibrium (with lateral deviation from normal orientation), and/or lordosis. These effects were only observed at the ≥0.271 mg ai/L levels and therefore were considered a result of treatment. The NOAEC and LOAEC for morphological and behavioral effects were 0.135 and 0.271 mg ai/L, respectively.

Table 8: Sub-lethal effects of AE C656948 (fluopyram) on fathead minnow between days 20 and 33. [(a)]

Treatment (mg ai/L) Measured (and nominal) concentrations	Labored respiration, max. % affected	Remaining at surface, max. % affected	Loss of equilibrium, max. % affected	Lordosis, max. % affected	Deformed mouth, max. % affected	Dark coloration, max. % affected	Ventral hematoma, max. % affected	Swollen belly, max. % affected
Control (dilution water only)	0	0	0	0	0	0	0	0
Solvent control	0	0	0	0	0	0	0	0
0.0176 (0.0185)	0	0	0	0	0	0	0	0
0.0380 (0.0370)	0	0	0	0	0	0	0	0
0.0647 (0.0740)	0	0	0	0	0	0	0	0
0.135 (0.148)	0	0	0	0	0	0	0	0
0.271 (0.296)	21	0	0	0	0	58	2	2
0.570 (0.592)	100	5	5	2	2	2	40	0
1.05 (1.18)	11	50	0	22	60	22	20	0
NOAEC	0.135 mg ai/L							
LOAEC	0.271 mg ai/L							

[(a)] Reviewer-calculated from raw data provided in Appendices F1 and F5 of the study report: maximum percent affected = (maximum number of fish exhibiting effect at given interval ÷ total number of surviving fish at corresponding interval) x 100; (day observed).

C. REPORTED STATISTICS:

Data that were statistically analyzed by the study author included 1) time to hatch, 2) hatching success, 3) larval survival on day 5, 4) fry survival on day 33, 5) the mean total length of surviving fish at study termination, and 6) the mean dry weight of surviving fish at study termination. Data for the replicate chambers for each concentration were grouped together for analysis. Replicate means were used for statistical analysis since each test chamber (aquarium) was an experimental unit based on the design of the test system.

The study author reported that negative and solvent control data were compared for each endpoint using a t-test. No significant differences were observed, and the study author pooled the data for subsequent comparisons. Data were analyzed for normal distribution using the R/s test procedure and for homogeneity of variances using Cochran's test. Time to hatch, hatching success, and fry survival (day 33) were arcsine transformed prior to analysis using Williams-t-Test. Larval survival (day 5) were arcsine transformed prior to analysis using the Bonferroni-Holm-U-Test. Dry weight data were analyzed without transformation using the Williams-t-Test, and length data were analyzed without transformation using the Bonferroni-Holm-U-Test.

The NOAEC and LOAEC were based on significance data. All analyses were performed by the study author using ToxRat Solutions GmbH statistical software, at a 95% confidence level. Mean-measured concentrations were used in the comparisons. The study author-reported NOAEC and LOAEC values are presented in the table below:

Parameter	**NOAEC, mg ai/L**	**LOAEC, mg ai/L**
Time to hatch	≥1.05 mg ai/L	>1.05 mg ai/L
Hatching success	≥1.05 mg ai/L	>1.05 mg ai/L
Larval survival (day 5)	0.570 mg ai/L	1.05 mg ai/L
Fry survival (day 33)	0.271 mg ai/L	0.570 mg ai/L
Length	0.135 mg ai/L	0.271 mg ai/L
Dry weight	0.271 mg ai/L	0.570 mg ai/L
Morphological/behavioral effects	0.135 mg ai/L	0.271 mg ai/L

III. REVIEWER'S EVALUATION:

A. DEVIATIONS FROM GUIDELINES: No major deviations from OPPTS 850.1400 were noted.

B. OTHER STUDY DEFICIENCIES:

1. Details regarding the brood stock (e.g., age, health, feeding) were not reported.

2. Water hardness was not monitored at all test levels.

C. VERIFICATION OF STATISTICAL RESULTS:

Statistical Method: The reviewer verified results for percent hatch (day 5), larval survival, fry survival, length, and dry weight. All data were confirmed to be normally distributed and the variances were homogeneous. The NOAEC and LOAEC values were determined using ANOVA, followed by Dunnett's or William's multiple comparison tests. For all endpoints, the negative control was compared to the solvent control using a t-test and no differences were detected; the negative control was subsequently used for all comparisons to treatment groups. Because there was no larval mortality in all but the highest treatment group, a one-sided t-test was used to compare survival in this group to the control. Statistical analyses were conducted using Toxstat statistical software. The NOAEC and LOAEC values for time to hatch and morphological/behavioral effects were visually verified.

Parameter	**NOAEC, mg ai/L**	**LOAEC, mg ai/L**
Time to hatch	≥1.05 mg ai/L	>1.05 mg ai/L
Hatching success	≥1.05 mg ai/L	>1.05 mg ai/L
Larval survival (day 5)	0.570 mg ai/L	1.05 mg ai/L
Fry survival (day 33)	0.271 mg ai/L	0.570 mg ai/L
Length	0.271 mg ai/L	0.570 mg ai/L
Dry weight	0.271 mg ai/L	0.570 mg ai/L
Morphological/behavioral effects	0.135 mg ai/L	0.271 mg ai/L

D. ADDITIONAL REVIEWER COMMENTS:

With the exception of body length, the reviewer's results agreed with the results obtained by the study author. When compared to the negative control (rather than the pooled control), the reviewer's analysis only detected significant reductions from control at the top two doses. However, based on the sensitivity of morphological/behavioral effects, the reviewer concluded the same study NOAEC and LAOEC as the study author.

All validity requirements were met. Specifically, control survival was >66% at hatch and ≥70% at study termination.

Test substance concentrations were determined at 0, 7, 14, 21, 28, and 33 days. Except during a brief diluter malfunction (described below), all measured concentrations were within 20% of mean values. Aqueous test samples were analyzed by direct-injection HPLC/UV.

A brief dilution malfunction occurred on Days 20 and 21, when an adequate quantity of AE C656948 was not delivered to the 0.0740 mg ai/L test vessels (21-day result of 0.0009 mg ai/L). Analytical sampling following remediation determined that concentrations returned to correct levels (23-day result of 0.0664 mg ai/L). The study author reasoned that as the NOAEC was determined to be higher than this treatment level, this deviation was not considered to have any affect on the results of the study.

TWA concentrations were reviewer-calculated (refer to associated Excel worksheet in Appendix II) using the following equation:

$$C_{TWA} = \frac{\left(\frac{C_1 + C_0}{2}\right)(t_1 - t_0) + \left(\frac{C_2 + C_1}{2}\right)(t_2 - t_1) + \left(\frac{C_{n-1} + C_2}{2}\right)(t_{n-1} - t_2) + \left(\frac{C_n + C_{n-1}}{2}\right)(t_n - t_{n-1})}{t_n}$$

where:
C_{TWA} is the time-weighted average concentration,
C_j is the concentration measured at time interval j (j = 0, 1, 2,.. n)
t_j is the number of hours (or days or weeks, units used just need to be consistent in the equation) of the test at time interval j (e.g., t_0 = 0 hours (test initiation), t_1 =24 hours, t_2 =96 hours).

Biomass loading was determined at the end of the test using the wet weights of the negative and solvent control fish. The mean wet weight was 157.6 mg. The biomass loading factor based upon the 3.75 L volume of a single growth chamber was 0.63 g/L (instantaneous) or 0.05 g/L/day.

The accuracy of the test solution divisions was checked prior to the test initiation and was within 10% of the nominal value. The diluter system and syringe pump function were checked each weekday during the test. Test solutions flowed through the diluter system for 6 days prior to study initiation.

Experimental test dates were May 18, 2006 to August 2, 2006.

E. CONCLUSIONS:

This study is scientifically sound/unsound and is thus acceptable/unacceptable. Exposure to AE C656948 (fluopyram) resulted in treatment-related reductions in larval survival (day 5) at the 1.05 mg ai/L level, fry survival (day 33) at the ≥0.560 mg ai/L levels, and length and dry weight of surviving fry at the >0.269 mg ai/L levels (TWA concentrations). Morphological and/or behavioral effects were observed at the ≥0.269 mg ai/L levels. No treatment-related effects on time to hatch or hatching success were indicated. Clinical signs of toxicity was the most sensitive endpoint, resulting in a NOAEC and LOAEC of 0.135 and 0.269 mg ai/L, respectively.

LOAEC: 0.269 mg ai/L
NOAEC: 0.135 mg ai/L

Endpoint(s) Affected: larval survival (day 5), fry survival (day 33), morphological and/or behavioral effects, and growth (length and dry weight)
Most sensitive endpoint(s): morphological and/or behavioral effects

III. REFERENCES:

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APPENDIX 1: OUTPUT OF REVIEWER'S STATISTICAL VERIFICATION:

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Egg Hatch (%) Day 5
File: 2336h        Transform: NO TRANSFORM

       t-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
-------------------------------------------------------------------------------
-
 GRP1 (SOLVENT CRTL) MEAN =    95.0000     CALCULATED t VALUE =     1.4942
 GRP2 (BLANK CRTL) MEAN   =    87.0000     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =     8.0000
-------------------------------------------------------------------------------
-
TABLE t VALUE (0.05 (2), 6) =  2.447    NO significant difference at
alpha=0.05
TABLE t VALUE (0.01 (2), 6) =  3.707    NO significant difference at
alpha=0.01

Egg Hatch (%) Day 5
File: 2336h       Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies
-------------------------------------------------------------------------------

INTERVAL     <-1.5     -1.5 to <-0.5     -0.5 to 0.5    >0.5 to 1.5    >1.5
             _____     _____________     ___________    ___________    _____

EXPECTED     2.144         7.744            12.224          7.744      2.144
OBSERVED     0            11                 8             13         0

-------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =  10.6839
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.



Egg Hatch (%) Day 5
File: 2336h       Transform: NO TRANSFORMATION

Shapiro Wilks test for normality
-------------------------------------------------------------------------------

D = 1004.000

W =    0.961

Critical W (P = 0.05) (n = 32) = 0.930
Critical W (P = 0.01) (n = 32) = 0.904
-------------------------------------------------------------------------------

Data PASS normality test at P=0.01 level. Continue analysis.



Egg Hatch (%) Day 5
File: 2336h        Transform: NO TRANSFORMATION
```

```
Hartley test for homogeneity of variance
--------------------------------------------------------------------------------

Calculated H statistic (max Var/min Var) =   27.67
Closest, conservative, Table H statistic =  249.0  (alpha = 0.01)

Used for Table H ==>     R (# groups) =   8,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   8,    df (# avg reps-1) =   3.00

--------------------------------------------------------------------------------

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal
      but do not differ greatly, the Hartley test may still be used
      as an approximate test (average df are used).



Egg Hatch (%) Day 5
File: 2336h       Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance
--------------------------------------------------------------------------------

Calculated B statistic =    8.27
Table Chi-square value =   18.48  (alpha = 0.01)
Table Chi-square value =   14.07  (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   3.00
Used for Chi-square table value ==>   df (#groups-1) =   7
--------------------------------------------------------------------------------

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).



Egg Hatch (%) Day 5
File: 2336h       Transform: NO TRANSFORMATION

                                ANOVA TABLE
--------------------------------------------------------------------------------

SOURCE             DF              SS                MS             F
--------------------------------------------------------------------------------
Between             7            131.500          18.786          0.449

Within (Error)     24           1004.000          41.833
--------------------------------------------------------------------------------
Total              31           1135.500
--------------------------------------------------------------------------------

  Critical F value =   2.42  (0.05,7,24)
```

Since F < Critical F FAIL TO REJECT Ho:All groups equal

Egg Hatch (%) Day 5
File: 2336h Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 1 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	TRANSFORMED MEAN	MEAN CALCULATED IN ORIGINAL UNITS	T STAT	SIG
1	neg control	95.000	95.000		
2	0.0175	89.000	89.000	1.312	
3	0.0380	90.000	90.000	1.093	
4	0.0652	91.000	91.000	0.875	
5	0.135	93.000	93.000	0.437	
6	0.269	92.000	92.000	0.656	
7	0.560	95.000	95.000	0.000	
8	1.05	92.000	92.000	0.656	

Dunnett table value = 2.48 (1 Tailed Value, P=0.05, df=24,7)

Egg Hatch (%) Day 5
File: 2336h Transform: NO TRANSFORMATION

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	0.0175	4	11.342	11.9	6.000
3	0.0380	4	11.342	11.9	5.000
4	0.0652	4	11.342	11.9	4.000
5	0.135	4	11.342	11.9	2.000
6	0.269	4	11.342	11.9	3.000
7	0.560	4	11.342	11.9	0.000
8	1.05	4	11.342	11.9	3.000

Egg Hatch (%) Day 5
File: 2336h Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	95.000	95.000	95.000
2	0.0175	4	89.000	89.000	91.714
3	0.0380	4	90.000	90.000	91.714
4	0.0652	4	91.000	91.000	91.714

5	0.135	4	93.000	93.000	91.714
6	0.269	4	92.000	92.000	91.714
7	0.560	4	95.000	95.000	91.714
8	1.05	4	92.000	92.000	91.714

Egg Hatch (%) Day 5
File: 2336h Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	95.000				
0.0175	91.714	0.718		1.71	k= 1, v=24
0.0380	91.714	0.718		1.79	k= 2, v=24
0.0652	91.714	0.718		1.82	k= 3, v=24
0.135	91.714	0.718		1.83	k= 4, v=24
0.269	91.714	0.718		1.84	k= 5, v=24
0.560	91.714	0.718		1.84	k= 6, v=24
1.05	91.714	0.718		1.85	k= 7, v=24

s = 6.468
Note: df used for table values are approximate when v > 20.

fry survival
File: 2336s Transform: NO TRANSFORM

t-test of Solvent and Blank Controls Ho:GRP1 MEAN = GRP2 MEAN

GRP1 (SOLVENT CRTL) MEAN = 96.5000 CALCULATED t VALUE = 0.9735
GRP2 (BLANK CRTL) MEAN = 93.2500 DEGREES OF FREEDOM = 6
DIFFERENCE IN MEANS = 3.2500

TABLE t VALUE (0.05 (2), 6) = 2.447 NO significant difference at alpha=0.05
TABLE t VALUE (0.01 (2), 6) = 3.707 NO significant difference at alpha=0.01

fry survival
File: 2336s Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies

INTERVAL	<-1.5	-1.5 to <-0.5	-0.5 to 0.5	>0.5 to 1.5	>1.5
EXPECTED	2.144	7.744	12.224	7.744	2.144

```
OBSERVED     0            10               13              9          0

------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   5.1982
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.


fry survival
File: 2336s       Transform: NO TRANSFORMATION

Shapiro Wilks test for normality
------------------------------------------------------------------------------

D = 2099.000

W =    0.963

Critical W (P = 0.05) (n = 32) = 0.930
Critical W (P = 0.01) (n = 32) = 0.904
------------------------------------------------------------------------------

Data PASS normality test at P=0.01 level. Continue analysis.


fry survival
File: 2336s        Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance
------------------------------------------------------------------------------

Calculated H statistic (max Var/min Var) =   22.07
Closest, conservative, Table H statistic =  249.0  (alpha = 0.01)

Used for Table H ==>     R (# groups) =   8,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   8,    df (# avg reps-1) =   3.00

------------------------------------------------------------------------------

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal
      but do not differ greatly, the Hartley test may still be used
      as an approximate test (average df are used).


fry survival
File: 2336s       Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance
------------------------------------------------------------------------------

Calculated B statistic =   10.05
```

```
Table Chi-square value =   18.48  (alpha = 0.01)
Table Chi-square value =   14.07  (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   3.00
Used for Chi-square table value ==>   df (#groups-1) =   7
---------------------------------------------------------------------------

Data PASS homogeneity test at 0.01 level. Continue analysis.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).


fry survival
File: 2336s        Transform: NO TRANSFORMATION

                              ANOVA TABLE
---------------------------------------------------------------------------

SOURCE             DF              SS                MS             F
---------------------------------------------------------------------------
Between             7           20414.500         2916.357         33.346

Within (Error)     24            2099.000           87.458
---------------------------------------------------------------------------
Total              31           22513.500
---------------------------------------------------------------------------

  Critical F value =   2.42  (0.05,7,24)
  Since  F > Critical F  REJECT  Ho:All groups equal



fry survival
File: 2336s        Transform: NO TRANSFORMATION

      DUNNETTS TEST   -   TABLE 1 OF 2             Ho:Control<Treatment
-------------------------------------------------------------------------
                            TRANSFORMED    MEAN CALCULATED IN
GROUP    IDENTIFICATION         MEAN          ORIGINAL UNITS    T STAT  SIG
----- --------------------    ----------    ------------------  ------  ---
  1            neg control      96.500             96.500
  2                 0.0175      91.500             91.500         0.756
  3                 0.0380      88.250             88.250         1.248
  4                 0.0652      93.250             93.250         0.491
  5                  0.135      89.750             89.750         1.021
  6                  0.269      86.750             86.750         1.474
  7                  0.560      70.000             70.000         4.007  *
  8                   1.05      15.000             15.000        12.325  *
-------------------------------------------------------------------------
Dunnett table value =  2.48     (1 Tailed Value, P=0.05,  df=24,7)



fry survival
File: 2336s        Transform: NO TRANSFORMATION
```

DUNNETTS TEST - TABLE 2 OF 2 Ho:Control<Treatment

GROUP	IDENTIFICATION	NUM OF REPS	Minimum Sig Diff (IN ORIG. UNITS)	% of CONTROL	DIFFERENCE FROM CONTROL
1	neg control	4			
2	0.0175	4	16.400	17.0	5.000
3	0.0380	4	16.400	17.0	8.250
4	0.0652	4	16.400	17.0	3.250
5	0.135	4	16.400	17.0	6.750
6	0.269	4	16.400	17.0	9.750
7	0.560	4	16.400	17.0	26.500
8	1.05	4	16.400	17.0	81.500

fry survival
File: 2336s Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	96.500	96.500	96.500
2	0.0175	4	91.500	91.500	91.500
3	0.0380	4	88.250	88.250	90.750
4	0.0652	4	93.250	93.250	90.750
5	0.135	4	89.750	89.750	89.750
6	0.269	4	86.750	86.750	86.750
7	0.560	4	70.000	70.000	70.000
8	1.05	4	15.000	15.000	15.000

fry survival
File: 2336s Transform: NO TRANSFORMATION

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	96.500				
0.0175	91.500	0.756		1.71	k= 1, v=24
0.0380	90.750	0.870		1.79	k= 2, v=24
0.0652	90.750	0.870		1.82	k= 3, v=24
0.135	89.750	1.021		1.83	k= 4, v=24
0.269	86.750	1.474		1.84	k= 5, v=24
0.560	70.000	4.007	*	1.84	k= 6, v=24
1.05	15.000	12.325	*	1.85	k= 7, v=24

s = 9.352
Note: df used for table values are approximate when v > 20.

```
length
File: 2336l        Transform: NO TRANSFORM

       t-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
-------------------------------------------------------------------------------
-
 GRP1 (SOLVENT CRTL) MEAN =    20.9000     CALCULATED t VALUE =    -0.2379
 GRP2 (BLANK CRTL) MEAN   =    21.0000     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =    -0.1000
-------------------------------------------------------------------------------
-
TABLE t VALUE (0.05 (2), 6) =  2.447    NO significant difference at
alpha=0.05
TABLE t VALUE (0.01 (2), 6) =  3.707    NO significant difference at
alpha=0.01



length
File: 2336l       Transform: NO TRANSFORMATION

Chi-square test for normality: actual and expected frequencies
-------------------------------------------------------------------------------

INTERVAL     <-1.5     -1.5 to <-0.5     -0.5 to 0.5    >0.5 to 1.5    >1.5
             _____     _____________     ___________    ___________    _____

EXPECTED     2.144         7.744            12.224          7.744      2.144
OBSERVED     0            10                13              9          0

-------------------------------------------------------------------------------
Calculated Chi-Square goodness of fit test statistic =   5.1982
Table Chi-Square value (alpha = 0.01) = 13.277

Data PASS normality test. Continue analysis.



length
File: 2336l       Transform: NO TRANSFORMATION

Shapiro Wilks test for normality
-------------------------------------------------------------------------------

D =   25.837

W =    0.807

Critical W (P = 0.05) (n = 32) = 0.930
Critical W (P = 0.01) (n = 32) = 0.904
-------------------------------------------------------------------------------

Data FAIL normality test. Try another transformation.
```

```
Warning -  The two homogeneity tests are sensitive to non-normal data and
           should not be performed.



length
File: 2336l        Transform: NO TRANSFORMATION

Hartley test for homogeneity of variance
--------------------------------------------------------------------------------

Calculated H statistic (max Var/min Var) =  198.97
Closest, conservative, Table H statistic =  249.0  (alpha = 0.01)

Used for Table H ==>     R (# groups) =   8,    df (# reps-1) =       3
Actual values    ==>     R (# groups) =   8,    df (# avg reps-1) =   3.00

--------------------------------------------------------------------------------

Data PASS homogeneity test. Continue analysis.

NOTE: This test requires equal replicate sizes. If they are unequal
      but do not differ greatly, the Hartley test may still be used
      as an approximate test (average df are used).



length
File: 2336l       Transform: NO TRANSFORMATION

Bartletts test for homogeneity of variance
--------------------------------------------------------------------------------

Calculated B statistic =   31.38
Table Chi-square value =   18.48  (alpha = 0.01)
Table Chi-square value =   14.07  (alpha = 0.05)

Average df used in calculation  ==>   df (avg n - 1) =   3.00
Used for Chi-square table value ==>   df (#groups-1) =   7
--------------------------------------------------------------------------------

Data FAIL homogeneity test at 0.01 level. Try another transformation.

NOTE: If groups have unequal replicate sizes the average replicate size is
      used to calculate the B statistic (see above).



dry weight
File: 2336w        Transform: NO TRANSFORM

       t-test of Solvent and Blank Controls          Ho:GRP1 MEAN = GRP2 MEAN
--------------------------------------------------------------------------------
-
 GRP1 (SOLVENT CRTL) MEAN =    35.1500     CALCULATED t VALUE =    -0.9219
 GRP2 (BLANK CRTL) MEAN   =    36.8250     DEGREES OF FREEDOM =     6
 DIFFERENCE IN MEANS      =    -1.6750
```

```
-------------------------------------------------------------------------
-
TABLE t VALUE (0.05 (2), 6) =  2.447    NO significant difference at
alpha=0.05
TABLE t VALUE (0.01 (2), 6) =  3.707    NO significant difference at
alpha=0.01



dry weight
File: 2336w        Transform: NO TRANSFORM

                              ANOVA TABLE
-------------------------------------------------------------------------

SOURCE             DF              SS                MS             F
-------------------------------------------------------------------------
Between             7            4597.842          656.835         86.848

Within (Error)     24             181.502            7.563
-------------------------------------------------------------------------
Total              31            4779.345
-------------------------------------------------------------------------

  Critical F value =   2.42  (0.05,7,24)
  Since  F > Critical F  REJECT  Ho:All groups equal



dry weight
File: 2336w        Transform: NO TRANSFORM

      DUNNETTS TEST   -   TABLE 1 OF 2            Ho:Control<Treatment
-------------------------------------------------------------------------
                              TRANSFORMED    MEAN CALCULATED IN
GROUP    IDENTIFICATION          MEAN          ORIGINAL UNITS    T STAT  SIG
----- --------------------    ----------    ------------------  ------  ---
  1            neg control      35.150             35.150
  2                 0.0175      39.500             39.500        -2.237
  3                 0.0380      41.500             41.500        -3.265
  4                 0.0652      38.050             38.050        -1.491
  5                  0.135      39.675             39.675        -2.327
  6                  0.269      36.500             36.500        -0.694
  7                  0.560      22.125             22.125         6.698  *
  8                   1.05       4.075              4.075        15.980  *
-------------------------------------------------------------------------
Dunnett table value =  2.48     (1 Tailed Value, P=0.05,  df=24,7)



dry weight
File: 2336w        Transform: NO TRANSFORM

      DUNNETTS TEST   -   TABLE 2 OF 2            Ho:Control<Treatment
-------------------------------------------------------------------------
                           NUM OF   Minimum Sig Diff  % of     DIFFERENCE
GROUP    IDENTIFICATION     REPS     (IN ORIG. UNITS)  CONTROL  FROM CONTROL
```

1	neg control	4			
2	0.0175	4	4.823	13.7	-4.350
3	0.0380	4	4.823	13.7	-6.350
4	0.0652	4	4.823	13.7	-2.900
5	0.135	4	4.823	13.7	-4.525
6	0.269	4	4.823	13.7	-1.350
7	0.560	4	4.823	13.7	13.025
8	1.05	4	4.823	13.7	31.075

dry weight
File: 2336w Transform: NO TRANSFORM

WILLIAMS TEST (Isotonic regression model) TABLE 1 OF 2

GROUP	IDENTIFICATION	N	ORIGINAL MEAN	TRANSFORMED MEAN	ISOTONIZED MEAN
1	neg control	4	35.150	35.150	38.775
2	0.0175	4	39.500	39.500	38.775
3	0.0380	4	41.500	41.500	38.775
4	0.0652	4	38.050	38.050	38.775
5	0.135	4	39.675	39.675	38.775
6	0.269	4	36.500	36.500	36.500
7	0.560	4	22.125	22.125	22.125
8	1.05	4	4.075	4.075	4.075

dry weight
File: 2336w Transform: NO TRANSFORM

WILLIAMS TEST (Isotonic regression model) TABLE 2 OF 2

IDENTIFICATION	ISOTONIZED MEAN	CALC. WILLIAMS	SIG P=.05	TABLE WILLIAMS	DEGREES OF FREEDOM
neg control	38.775				
0.0175	38.775	1.864	*	1.71	k= 1, v=24
0.0380	38.775	1.864	*	1.79	k= 2, v=24
0.0652	38.775	1.864	*	1.82	k= 3, v=24
0.135	38.775	1.864	*	1.83	k= 4, v=24
0.269	36.500	0.694		1.84	k= 5, v=24
0.560	22.125	6.698	*	1.84	k= 6, v=24
1.05	4.075	15.981	*	1.85	k= 7, v=24

s = 2.750
Note: df used for table values are approximate when v > 20.

APPENDIX II: COPY OF REVIEWER'S TWA CALCULATIONS:

Time-Weighted Concentrations

Nominal (mg ai/L)	Time (Day)	Mean Measured Concentration (mg ai/L)	TWA (mg ai/L)
0.0185	0	0.0178	
	7	0.0162	
	14	0.0177	
	21	0.0176	
	28	0.0182	
	33	0.0178	
	TWA		**0.0174697**
0.037	0	0.0363	
	7	0.0392	
	14	0.0385	
	21	0.0374	
	28	0.0375	
	33	0.0392	
	TWA		**0.038053**
0.074	0	0.0589	
	7	0.0633	
	14	0.069	
	23	0.0664	*
	28	0.0651	
	33	0.0653	
	TWA		**0.065297**
0.148	0	0.129	
	7	0.136	
	14	0.147	
	21	0.128	
	28	0.132	
	33	0.138	
	TWA		**0.1353182**
0.296	0	0.267	
	7	0.268	
	14	0.272	

	21		0.266	
	28		0.266	
	33		0.288	
		TWA		**0.2694697**
0.592	0		0.619	
	7		0.47	
	14		0.552	
	21		0.58	
	28		0.6	
	33		0.599	
		TWA		**0.5599394**
1.18	0		0.957	
	7		1.01	
	14		1.15	
	21		0.996	
	28		1.08	
	33		1.1	
		TWA		**1.0506515**

*Short-term malfulction on Day 21; data not included in TWA calculation